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Influence of Surface-Imprinted Nanoparticles on Trypsin Activity

Dr. António Guerreiro[*],
Department of Chemistry, University Road, Leicester, LE1 7RH, UK

Dr. Alessandro Poma,
Department of Chemistry and Analytical Sciences, Open University, Milton Keynes, MK7 6AA, UK

Dr. Kal Karim,
Department of Chemistry, University Road, Leicester, LE1 7RH, UK

Dr. Ewa Moczko,
Department of Chemistry, University Road, Leicester, LE1 7RH, UK

Jessica Takarada,
Instituto de Ciências Exatas, Universidade Federal de Alfenas, Alfenas, MG, 37130-000, Brazil

Dr. Isabel Perez de Vargas-Sansalvador,
Cranfield Health, Cranfield University, MK43 0AL, UK

Dr. Nicholas Turner,
Department of Chemistry and Analytical Sciences, Open University, Milton Keynes, MK7 6AA, UK

Dr. Elena Piletska,
Department of Chemistry, University Road, Leicester, LE1 7RH, UK

Dr. Cristiana Schmidt de Magalhães,
Instituto de Ciências Exatas, Universidade Federal de Alfenas, Alfenas, MG, 37130-000, Brazil

Dr. Natalia Glazova,
SBEI HVE Saint-Petersburg State Chemical Pharmaceutical Academy of the Ministry of Health, 197376, Russian Federation

Dr. Anastasia Serkova,
SBEI HVE Saint-Petersburg State Chemical Pharmaceutical Academy of the Ministry of Health, 197376, Russian Federation

Aleksandra Omelianova, and
SBEI HVE Saint-Petersburg State Chemical Pharmaceutical Academy of the Ministry of Health, 197376, Russian Federation

Prof. Sergey Piletsky
Department of Chemistry, University Road, Leicester, LE1 7RH, UK

[*]ag398@leicester.ac.uk.

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Molecularly Imprinted Polymer Nanoparticles (MIP NPs) were synthesized via a solid-phase approach with immobilized template enzyme (trypsin) on a solid support which following polymer synthesis acted as affinity matrix for separation of high-affinity nanoparticle fractions. This protocol was adapted from Poma *et al*.[1] and allows for fast synthesis and controlled separation and purification of high affinity materials, with one production cycle lasting just 2.5 hours. Materials produced this way were free from template contamination and possessed sub-nanomolar apparent dissociation constants whilst showing low cross-reactivity. Depending on the enzyme immobilization method (random vs. oriented, with protected enzyme active site) used during imprinting, the rebinding of the free enzyme onto the MIP NP results either in its inhibition or in apparent stabilization (no inhibition observed).

MIPs can be broadly considered as man-made equivalents of natural receptors/antibodies, and, like their natural counterparts are able to recognize and bind corresponding target molecules. For this reason, and also due to their potential to replace unstable natural receptors in diagnostics, MIPs are an important research target.[2-4] When used in nanoparticle format, as opposed to more “traditional” approaches such as ground monoliths, beads or films, MIPs have the potential to be used as direct replacement for natural receptors (such as antibodies) in assays, sensing and affinity separations[3] and catalysis.[5] While the majority of work performed with imprinted polymers deals with small molecules, imprinting of proteins remains challenging [6] due to their high molecular-weights, as well as multitude of functional groups which together with low stability in non-physiological conditions hinder the imprinting process. This fact, together with the lack of a scalable and standard process for the synthesis of MIP NPs restricts access of this technology to prospective practical and commercial applications.

Recently, solid-phase imprinting has emerged as a promising new route for the manufacture of high-affinity MIP NPs, being simple, easy to implement and even automate. [1, 7] Unlike traditional protocols, where the template is free in solution, this methodology relies on immobilized template on the surface of a solid support (in present case, glass beads with average diameter of 90 μm), which are placed in the polymerization mixture. Precipitation polymerization is then initiated chemically; the whole process is performed in mild aqueous conditions, suitable for protein imprinting. The low concentration of monomers (6.5 mM total monomer concentration) ensures no macro polymer is formed, and materials are obtained in nanoparticle format. After polymerization, the solid phase with surface immobilized template functions as an affinity support for purification of high affinity NPs, see Scheme 1.

In order to produce MIP NPs with affinity for trypsin using the solid-phase approach, a mixture of monomers previously used to imprint large biomolecules in aqueous conditions was adapted from,[8, 9] see Scheme 1 and S.I. for full details. Different monomers were used

in order to provide a range of ionic and hydrophobic interactions, in addition to hydrogen bonds, between monomers and the large protein template. For random template immobilization, trypsin was covalently attached on the surface of amine-silanated glass beads (solid phase) via glutaraldehyde coupling. Silanization of glass surfaces measured by Sheng[10] for glutaraldehyde modified (3-aminopropyl) trimethoxysilane (as used in this work) gave probe densities of 1 site per 3.8 nm^2. Taking into account the size of trypsin (3.8×3.8×3.8 nm),[11] it is plausible to assume an even surface coverage of the glass beads used as solid support. The amount of trypsin immobilized was 0.04 mg $gram^{-1}$ of glass beads.

An additional advantage of having the template immobilized is the possibility to imprint oriented proteins. Oriented immobilization was achieved by first immobilizing a trypsin inhibitor (aprotinin), via glutaraldehyde coupling, onto the glass beads, then incubating the beads containing inhibitor with trypsin. The amount of trypsin immobilized this way was 0.03 mg $gram^{-1}$ of glass beads. After immobilization of the template, the beads were placed in the monomer solution and polymerization initiated. Imprinted polymer is formed around the immobilized template and so remains attached to the solid phase due to affinity interactions. Polymer formed in solution, with improperly formed imprints and unreacted monomer will naturally have lower affinity and are removed during the subsequent washing step, see Scheme 1. Additional advantages of this process include: narrow (monoclonal) distribution of binding site affinities,[7] high binding site accessibility, possibility to imprint whole proteins and in theory any synthetic epitope peptide;[12] MIP NPs can be collected as a pure fraction, free from template and monomers by a temperature controlled elution; short synthesis and purification time of just 2.5 hours/batch, as compared to days needed with methods which rely on dialysis for template removal.[9] The yield of high-affinity nanoparticles obtained was 43 ± 3 % weight with respect to initial monomer mass, for 60 g of trypsin-derivatized solid phase and 50 mL of polymerization mixture prepared as described in S. I. No protein contamination was observed on high-affinity NP fractions using the BCA method, which has a detection limit of 5 μg mL^{-1}. Accordingly, no enzymatic activity was observed on any of the NP solutions. Dry particle size was ca. 80-100 nm, Scheme 1. Hydrodynamic size was assessed by Dynamic Light Scattering (DLS), with 207±12 nm obtained for MIP NP produced with randomly-immobilized trypsin (R-MIP) and 159±7 nm for MIP obtained with oriented template (O-MIP); size distribution and SEM image presented in S.I. The difference between dry sizing and DLS can possibly be attributed to swelling of the low-crosslinked nanogel in water.

The affinity and selectivity of trypsin MIP NPs were assessed by Surface Plasmon Resonance (SPR) with immobilized enzyme template (Figure 1), this method was adapted from.[9] The calculated apparent dissociation constant (K_d) was 5.5 pM for trypsin R-MIP NP injected on trypsin surface (Figure 1), calculated using 1:1 Langmuir binding model, with a good correlation between the model and experimental data (chi^2 of 0.1, where values close to zero represent accurate data fit). On control experiments, (trypsin R-MIP on pepsin surface) no binding equilibrium or saturation could be achieved even at the highest concentration of nanoparticles tested (0.9 nM), which implies a K_d above 0.9 nM. Increasing concentration above this value results in formation of particle aggregates, with appearance

of slight opalescence in solution, and so unsuitable for SPR analysis. K_d obtained for O-MIP NP was 2.8 pM, with a chi^2 of 0.06 using a 1:1 Langmuir binding model, Figure 1. All K_d were calculated with BiaEvaluation software v4.1.

The low K_d obtained can be explained by a large number of weak interactions occurring at various points (in the imprinted binding cavity) between the polymer and the large template molecule, having a significant cumulative effect which ultimately results in high affinity. It is however worth pointing out that the calculated K_d's are "apparent", intended mainly to compare/verify the relative affinities of the particles to the different targets under similar conditions. So, should not be considered as an "absolute" value for this type of material, which might differ under different circumstances, such as that for immobilized particles or for free target/particles in solution.

As can be seen in Figure 1, (on the right) when O-MIP NP were injected on the SPR chip with randomly immobilized trypsin, surface saturation was reached earlier, and lower responses were obtained, when compared with R-MIP NPs injected on this same chip surface. This can be attributed to the presence of a smaller fraction of enzyme molecules in the right orientation at the chip surface (due to random immobilization), with consequent reduction in the actual number of particles bound to the surface.

Soluble polymeric nanoparticles which specifically bind to targets of medical interest (such as enzymes), much like an "artificial antibody" are good candidates to replace small molecule and antibody-based therapies [13, 14] as enzyme modulators. In order to assess if the high-affinity particles possessed biological activity, modulating the activity of the enzyme used as template, trypsin was incubated with either control (non-trypsin imprinted) C-NPs or R-MIP NPs, and its activity compared with that of free enzyme. As no non-imprinted materials can be produced using this approach (because the affinity separation step cannot be performed in the absence of template), control (C-NPs) were produced using an immobilized glycopeptide (vancomycin), and bovine serum albumin (BSA) as template, monomer composition was in both cases similar to that used with trypsin. Two templates were used as to verify if NP imprinted with large BSA molecules (and consequently possessing a larger binding cavity), will have any effect on the unspecific binding of trypsin to the NP, also, in the case of vancomycin, the aim was to produce particles which are close to classical "non-imprinted" materials, by using a comparatively small template. DLS size was 150 ± 4.8 and 166 ± 5 nm for vancomycin and BSA C-NPs, respectively. Trypsin was selected as demonstration target as it was the object of recent similar studies where imprinting was aided by the inclusion of a polymerizable inhibitor monomer (benzamidine derivative) during MIP synthesis. In absence of the polymerizable inhibitor, enzyme imprinting was not possible.[13, 15] Here we performed MIP synthesis without resourcing to use of polymerizable inhibitors, a strategy we believe is more flexible and has broad applicability to proteins in general. When trypsin was incubated with R-MIP NPs, we observed enzyme inhibition. Results of inhibition studies performed in solution in the presence and absence of R-MIP NPs were plotted as Lineweaver–Burk graphs, Figure 2.

Inhibition constant K_i for mixed inhibition was obtained from Michaelis-Menten plots, using inhibitor concentration as NP molarity. For this, reciprocal apparent velocities (V_{app}) were

plotted against inhibitor concentration and the constant obtained at the intercept when $1/V_{app} = 0$. Obtained inhibition constant was 19 nM. Compared with results obtained by Cutivet,[13] which imprinted trypsin using a polymerizable inhibitor, the inhibition constant obtained here was within the same order of magnitude (*vs*. 79 nM as obtained by Cutivet). However, in both cases, imprinted polymers are a more effective inhibitor than free benzamidine (small molecule inhibitor) which in solution has a K_i of 18.9 μM,[13] but less effective than aprotinin, with a K_i of 0.06 pM.[16] It must be noted that due to the randomly immobilized enzyme used during synthesis, some of the binding interactions between NP-enzyme will be non-productive, i.e. taking place in areas of the enzyme which are not necessarily linked with inhibition. Also, the actual molar concentration of the inhibiting fraction of NPs in the mixture used (containing both inhibiting and non-inhibiting NPs) can naturally be expected to be much lower than that of the total molar concentration of NPs as used for the determination of K_i. Both facts mentioned above will result in calculated K_i's which are higher than corresponding K_d's and once more, these are "apparent" values, for the total molar concentration of NPs mixture (as used) consisting of inhibiting and non-inhibiting particles.

Batch inhibition experiments were performed to compare the effects of R- and O-MIP on free trypsin in solution, for this the enzyme was incubated with either type of nanoparticles and its activity then assessed. C-NPs were used on control experiments, at the same concentration as the R- and O-MIP NPs. NP concentration was 1.8 nM, tests were performed in triplicate. In presence of the R-MIP, trypsin displayed 53.5 ± 4.9% remaining activity whilst in presence of either vancomycin or BSA C-NPs activity was mostly unaffected, with more than 99 % remaining. In contrast, when trypsin was incubated with O-MIP NPs, no inhibitory effects were observed. In this case it is expected that particles bound to the surface-imprint (on the O-MIP NP) will have the active site exposed, and this can possibly lead to increased enzyme stability, without affecting its activity. In order to assess this hypothesis, trypsin (10 μg) was then incubated with 1 mL of either O-MIP or C-NP, both at 1.8 nM, activity was then measured at 10 min and after 72 h. Tests were carried out at 22 and 4 °C. No change in enzyme activity could be detected after 10 min, for free enzyme (without NPs), C and O-MIP NPs. Afterwards; the results clearly show an increase in enzyme stability. At 22 °C, in absence of O-MIP and in presence of C-NP and enzyme alone, only 40 ± 3.6 % enzymatic activity remained after 72 h, whilst with O-MIP the value was 50 ± 3.0 %. At 4 °C, 48 ± 5.0 % of activity was recorded in the absence of MIP and in presence of C-NP and enzyme alone, whilst no loss in activity (100 ± 3.6 % remaining) was recorded in presence of O-MIP NPs. Stabilization of the enzyme, in this specific case can be attributed to two factors, stabilization of the protein structure (increase in protein structure rigidity in presence of MIP NPs was observed by circular dichroism, see S. I. for spectra) and prevention of self-digestion. In conclusion, MIP NPs were produced using a solid-phase approach which relies on template immobilized on a solid support for synthesis of MIP NPs, this support doubles as affinity matrix for selection and purification of particles after synthesis. This method offers significant advantages when compared to traditional approaches which rely on free template in solution, such as short synthesis/purification times, particles possess high affinity and specificity and are obtained as a "clean" fraction, free from monomers and template. Depending on template immobilization method, enzyme

rebinding to NPs leads to its inhibition (when randomly immobilized enzyme is used as template) or to enzyme stabilization (when oriented immobilized enzyme is used as template). This implies the formation of high quality imprints on the MIP NPs, capable of recognizing the orientation of the template protein. The results presented point towards the possibility of developing new drugs based on this type of materials, this can either be as scavengers/antidotes, or enzyme effectors (inhibitors or stabilizers capable of extending the lifetime/shelf life of enzymes and enzyme-based products). Future prospects include the imprinting of enzyme epitopes located near the active site; this has the potential to overcome the difficulties in immobilizing enzymes with an exposed active site, and so generate more potent inhibitory MIP NPs. Alternatively, particles could conceivably be used as replacement of natural receptors in sensing and separations, especially for demanding applications in harsh environments where protein-based materials might encounter stability problems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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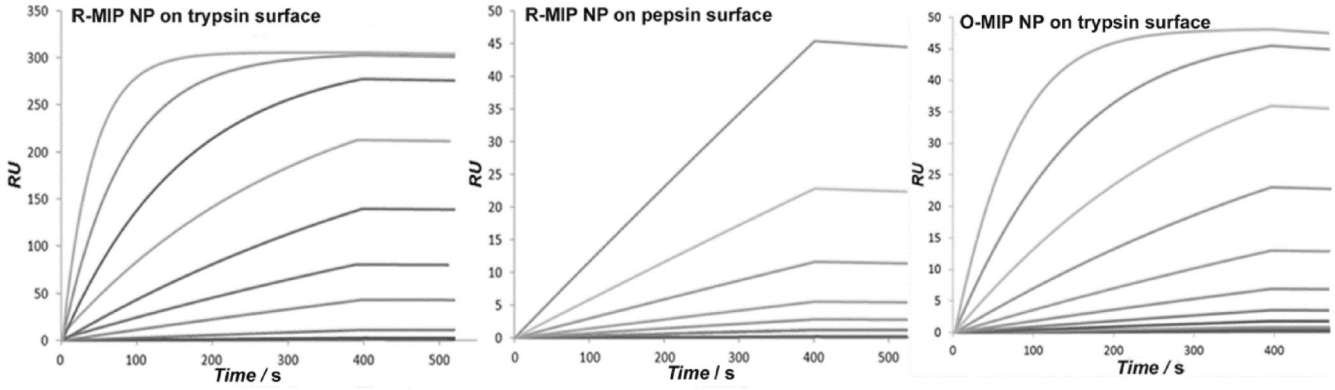

Figure 1.

SPR sensorgrams for trypsin MIP NPs (left) injected on trypsin coated sensor surface. Cross-reactivity (control) was assessed by injection of trypsin MIP NPs on a pepsin surface. Concentration of trypsin R-MIP NP ranged from 0.1 pM to 0.9 nM. On the right, SPR sensorgrams for oriented trypsin (O-MIP) NPs injected on trypsin coated sensor surface (randomly immobilized). Solutions of trypsin nanoparticles were injected at concentrations ranging from 0.26 pM to 0.28 nM. SPR tests were performed in PBS buffer pH 7.2, full details in S.I.

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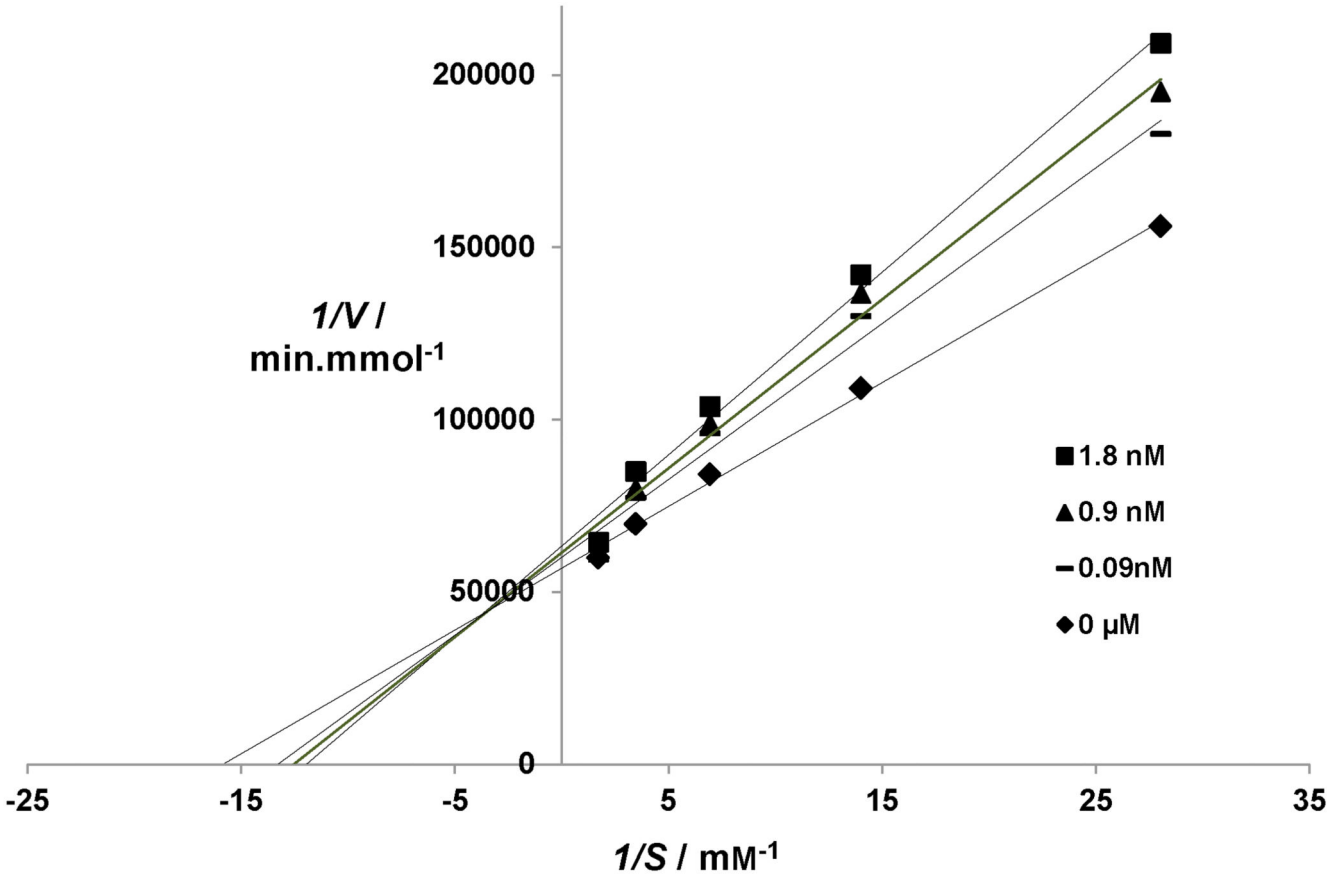
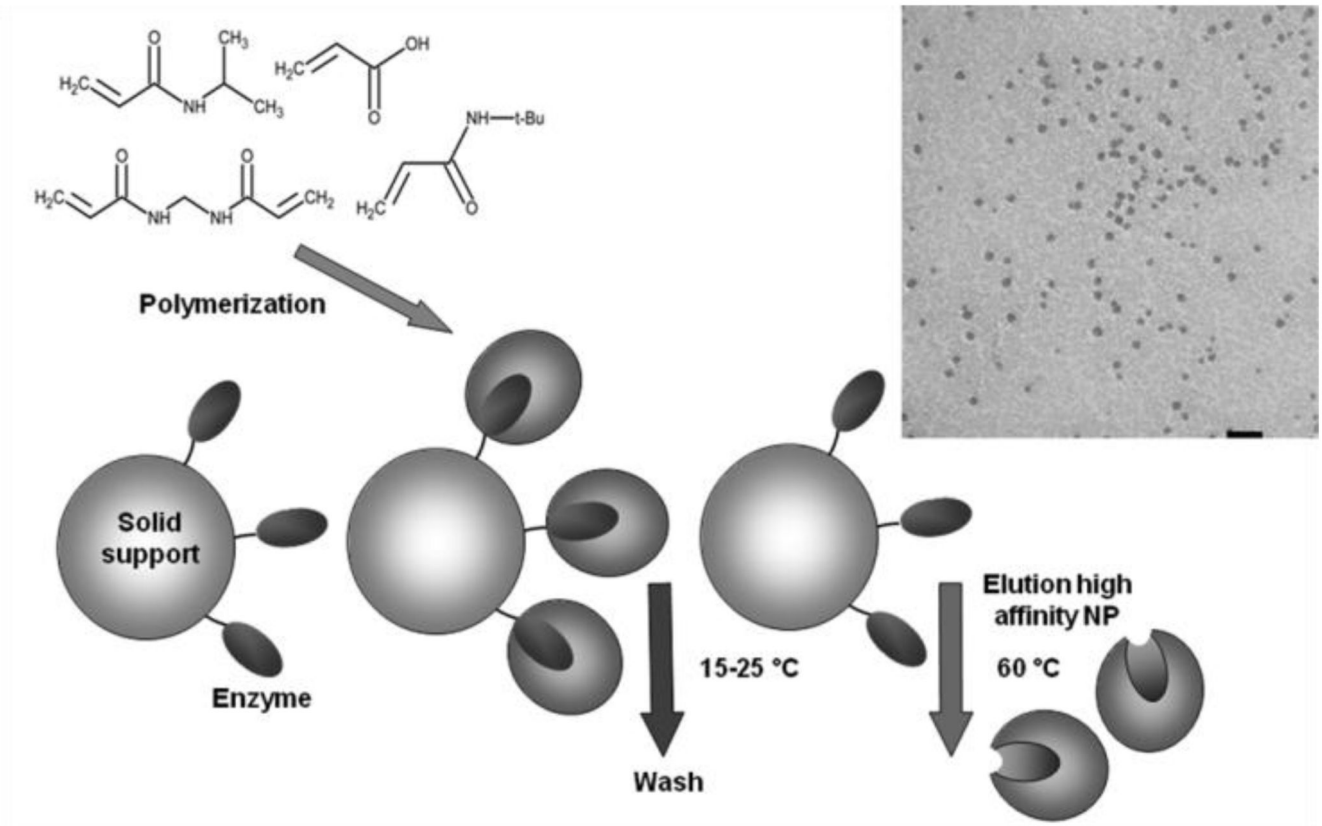


Figure 2.

Mixed trypsin inhibition by various concentrations of R-MIP NP and respective Lineweaver–Burk plots. Enzyme concentration was 10 µg mL^{-1}, substrate used was N_{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride, concentration ranged from 0.07 to 0.57 mM.



Scheme 1.

Schematic representation of the imprinting and purification process for MIP NP and TEM image of trypsin R-MIP NP at 20K magnification, scale bar represents 300 nm. Template (trypsin) is immobilized on the solid-phase (support) and then aqueous polymerization mixture is added. Monomers used were *N*-isopropylacrylamide, acrylic acid, *N*,*N*′-methylenebisacrylamide, *N*-tert-butylacrylamide. After initiation/polymerization a washing step is performed to remove low-affinity material and monomers, high-affinity NPs remain attached to the template. These are then eluted as a pure fraction by increasing the temperature of the reactor.

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